Post-translational modifications of Hsp70 family proteins: Expanding the chaperone code

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Cells must be able to cope with the challenge of folding newly synthesized proteins and refolding those that have become misfolded in the context of a crowded cytosol. One such coping mechanism that has appeared during evolution is the expression of well-conserved molecular chaperones, such as those that are part of the heat shock protein 70 (Hsp70) family of proteins that bind and fold a large proportion of the proteome. Although Hsp70 family chaperones have been extensively examined for the last 50 years, most studies have focused on regulation of Hsp70 activities by altered transcription, co-chaperone "helper" proteins, and ATP binding and hydrolysis. The rise of modern proteomics has uncovered a vast array of post-translational modifications (PTMs) on Hsp70 family proteins that include phosphorylation, acetylation, ubiquitination, AMPylation, and ADP-ribosylation. Similarly to the pattern of histone modifications, the histone code, this complex pattern of chaperone PTMs is now known as the "chaperone code." In this review, we discuss the history of the Hsp70 chaperone code, its currently understood regulation and functions, and thoughts on what the future of research into the chaperone code may entail.

The heat shock protein 70 (Hsp70) family consists of well-conserved yet functionally diverse molecular chaperones that are critical for nascent protein folding (1), clearance of misfolded/unfolded proteins (2), prevention of stress-induced protein aggregation (3), disaggregation of existing protein deposits, protein degradation (4), and chaperone-mediated autophagy (5). Mutations in genes encoding components of the Hsp70 system are linked to several human diseases, including Parkinson's disease (6–9), diabetes mellitus (10), colorectal cancer (2, 11) and cardiomyopathy (12, 13). As an integral part of the cellular proteostasis machinery, Hsp70s are critical for maintaining cell viability in response to a large variety of cellular stresses, including high temperature, nutrient starvation, osmotic shock, oxidative stress, and DNA damage (2). Historically, studies on Hsp70s have primarily focused on intrinsic folding activity driven by the binding and hydrolysis of ATP, interaction with helper co-chaperones, and inducibility of expression under stress. More recently, evidence has accumulated that Hsp70s are highly modified at the post-translational level (14–18). These modifications fine-tune chaperone function, altering chaperone activity, localization, and selectivity. In the same way that modifications on histones are collectively called the "histone code," we now refer to the complex array of post-translational modifications (PTMs) on chaperones as the "chaperone code." In this review, we summarize in detail the current knowledge of how PTMs contribute to the regulation of Hsp70 family chaperones. We further offer a perspective on future directions and challenges the field may encounter in establishing and integrating the physiological impact of the Hsp70 chaperone code.

The Hsp70 family of proteins is evolutionarily conserved and found in archaebacteria, prokaryotes, and eukaryotes (including plants and animals), establishing it as a crucial protein family in the phylogenetic tree of life (19–21). Hsp70s have been widely studied both *in vitro* and *in vivo*, using a combination of purified recombinant proteins, tissue culture setups, and model organism such as *Saccharomyces cerevisiae* (budding yeast). A testament to the high level of functional conservation is the ability of Hsp70s from diverse organisms (human, sea anemone, and plant) to maintain the cell viability of budding yeast when expressed as the sole cytosolic Hsp70 (22). *S. cerevisiae* contains seven cytosolic Hsp70 isoforms: the four canonical chaperones Ssa1, Ssa2, Ssa3, and Ssa4 and the three ribosome-associated chaperones Ssb1, Ssb2, and Ssz1. In addition, there are three mitochondrial isoforms (Ssc1, Ssq1, and Ecm10) and one specific to endoplasmic reticulum (Kar2) (23). Ssa1 and Ssa2 are constitutively expressed, whereas Ssa3 and Ssa4 are not present during normal growth but up-regulated in response to stress and in the stationary phase (24–27). Yeast mutants lacking all four canonical Hsp70 chaperones are not viable (28). Ssa1–4 are partially functionally redundant, with overexpression of any single Ssa isoform enough to provide cell viability in an *ssa1–4*Δ strain (22).

Human Hsp70s are encoded by a multigene family, which constitutes 17 genes and 30 pseudogenes (29). This multigene family gives rise to 13 gene products, which vary in their location, amino acid composition, and expression levels in the cell. Hsp70-1 (HSPA1A, Hsp70) and Hsp70-2 (HSPA1B, Hsp70) are the two major stress-inducible isoforms, which only differ from each other by two amino acids. The noninducible Ssz-like chaperone Hsp70-1t (HspA1L) is constitutively expressed and exhibits 90% identity to Hsp70-1. Hsp70-5 (HSPA5/BiP/GRP78) is localized to the endoplasmic reticulum and facilitates transport and folding of nascent polypeptides into the ER lumen. Hsp70-6 (HspA6) is an additional stress-inducible Hsp70 family member that has 85% homology to Hsp70-1 and

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is expressed in moderate levels in dendritic cells, monocytes, and natural killer cells but has no detectable basal expression level in other cells. HSPA7 has been considered a pseudogene transcribed in response to stress. Hsp70-8 (HSPA8, Hsc70, Hsp73, Hsc72) is the cognate Hsp70 family member that exhibits essential housekeeping functions such as folding of nascent polypeptides and misfolded proteins. Hsp70-9 (HSPA9, mortalin, GRP75, mtHsp70) is a mitochondrial Hsp70 isoform that bears a 46-amino acid target signal responsible for localization to the mitochondrial lumen (30, 31). Global knockouts of constitutively expressed Hsp70 isoform HSPA5, HSPA8, or HSPA9 are lethal, highlighting the central role of these chaperones in cellular physiology (32, 33).

Structural features of Hsp70 proteins

Structurally, Hsp70s consists of a 45-kDa N-terminal nucleotide-binding domain (NBD) and a 28-kDa substrate-binding domain (SBD) that are connected by a flexible linker (Figs. 1 and 2) (34, 35). The NBD consists of four subdomains (IA, I, IIA, and IIB), which are required for the binding and hydrolysis of ATP to ADP. The SBD is further subdivided into a 15-kDa substrate-binding domain (SBDβ) and a 10-kDa helical lid domain (SBDα) acting as a flexible lid. The hydrolysis of ATP promotes NBD-conformational changes, which are transduced through the linker domain and trigger the clamping down of the lid domain onto unfolded protein clients, preventing substrate dissociation and providing an opportunity for the protein to obtain its native fold (1). The release of ADP and subsequent binding of fresh ATP promotes the release of the folded substrate, setting Hsp70 up for the next round of folding.

Regulation of Hsp70 function

The activity of Hsp70 family chaperones is tightly regulated. A first layer of regulation is provided by the rapid expression of stress-inducible Hsp70 isoforms in the presence of protein-unfolding stress. The up-regulation of Hsp70-1 and Hsp70-2 in the cytoplasm and BiP in the ER are key events during the induction of cellular stress responses, such as the heat shock response or the unfolded protein response in the ER (UPR^{ER}). These compartment-specific Hsp70 enrichments provide cells with enhanced protein (re)folding capacities to prevent or resolve stress-induced damage. Historically, Hsp70 was thought to exist primarily in a monomeric state, but recent evidence suggests that Hsp70 can form monomers, dimers, trimers, and higher-order oligomers (36). Although the functions of these high-order forms have not been fully delineated, they are clearly required for at least a subset of chaperone functions (36–38). The Hsp70 folding cycle is accelerated and regulated by a suite of co-chaperone proteins, including Hsp40s and nucleotide exchange factors (NEFs). Hsp40s are a heterogenous family of co-chaperones bearing a conserved J-domain required for binding to Hsp70 (39). These co-chaperones can bind to unfolded proteins via their C terminus to prevent aggregation and transport them to Hsp70 for folding/refolding. Hsp40s also play a more direct role in regulating Hsp70 by directly stimulating the ATP hydrolysis activity of Hsp70, locking the client into the closed SBD (39, 40). Once a client protein is folded, NEFs facilitate ADP release and mediate the exchange of ADP with ATP, which is required for the opening of the lid and the release of the folded client from the SBD (34, 41–45). Another class of co-chaperones that regulate Hsp70 activity includes the tetratricopeptide repeat proteins Hip, Hop, and CHIP. Hip prevents the dissociation of ADP from Hsp70 binding to its NBD (46, 47). Hop coordinates with both Hsp70 and Hsp90 and targets Hsp90 to the Hsp70-client protein complex (48–50). CHIP acts as an E3 ubiquitin ligase, ubiquitinating Hsp70 substrates, which results in their degradation by the proteasome (51). The number of highly related co-chaperones (*e.g.* 13 NEFs and 41 J-domain–containing proteins in humans) substantially exceeds the number of Hsp70 isoforms, consistent with the idea that they are critically involved in regulating the functional diversity of Hsp70s (3).

History of the Hsp70 chaperone code

Early studies that identified modification of Hsp70 were generally descriptive in nature. PTMs, such as phosphorylation, were detected on chaperones, yet the sites of modification, their regulation, and their function remained elusive (52–54). A decrease in cost and increase in the resolution of proteomic experiments led to an abundance of PTMs being discovered on Hsp70s. In 2012, bioinformatic attempts to characterize regions of functionally important PTMs on proteins ("hotspots") identified two such hotspots on Hsp70 (55). The first region was located in the NBD (Thr-36 and Thr-38), whereas the second is present on the SBD (Thr-492, Ser-495, and Thr-499). Mutation of these sites led to viable cells that exhibited compromised chaperone function, including an inability to refold denatured proteins and increased global protein aggregation (55). The first mechanistic study of an Hsp70 PTM was published later in 2012, when it was demonstrated that a conserved site on Hsp70 was phosphorylated by cell cycle kinases, leading to altered cyclin stability (56). Follow-up studies demonstrating that both C-terminal phosphorylation and methylation of Hsp70 can impact chaperone function led to the proposal of the "chaperone code" (14, 57, 58). In this model, multiple cellular signals converge on Hsp70 (and indeed other chaperones such as Hsp90), leading to the fine-tuning of chaperone interactions and altered flow of information through cellular pathways.

More recently, bioinformatics-based approaches, molecular weight shift analysis, isoelectric properties of protein, isotope labeling, and high-resolution MS have been used to uncover many more PTMs on the Hsp70 family of proteins, including acetylation, methylation, ubiquitination, SUMOylation, AMPylation, and ADP-ribosylation. Thankfully, several online resources facilitate tracking and interpreting the potential impact of Hsp70 PTMs. PhosphositePlus® (RRID:SCR_001837) is a comprehensive and curated online resource that collects information regarding the target proteins/residues and functional implications of abundant PTMs, such as phosphorylation, acetylation, methylation, ubiquitination, and *O*-glycosylation (59). The Global Proteome Machine Database (RRID:SCR_006617) is another excellent resource and is updated on a daily basis (60). Analysis of the available data from sites such as these

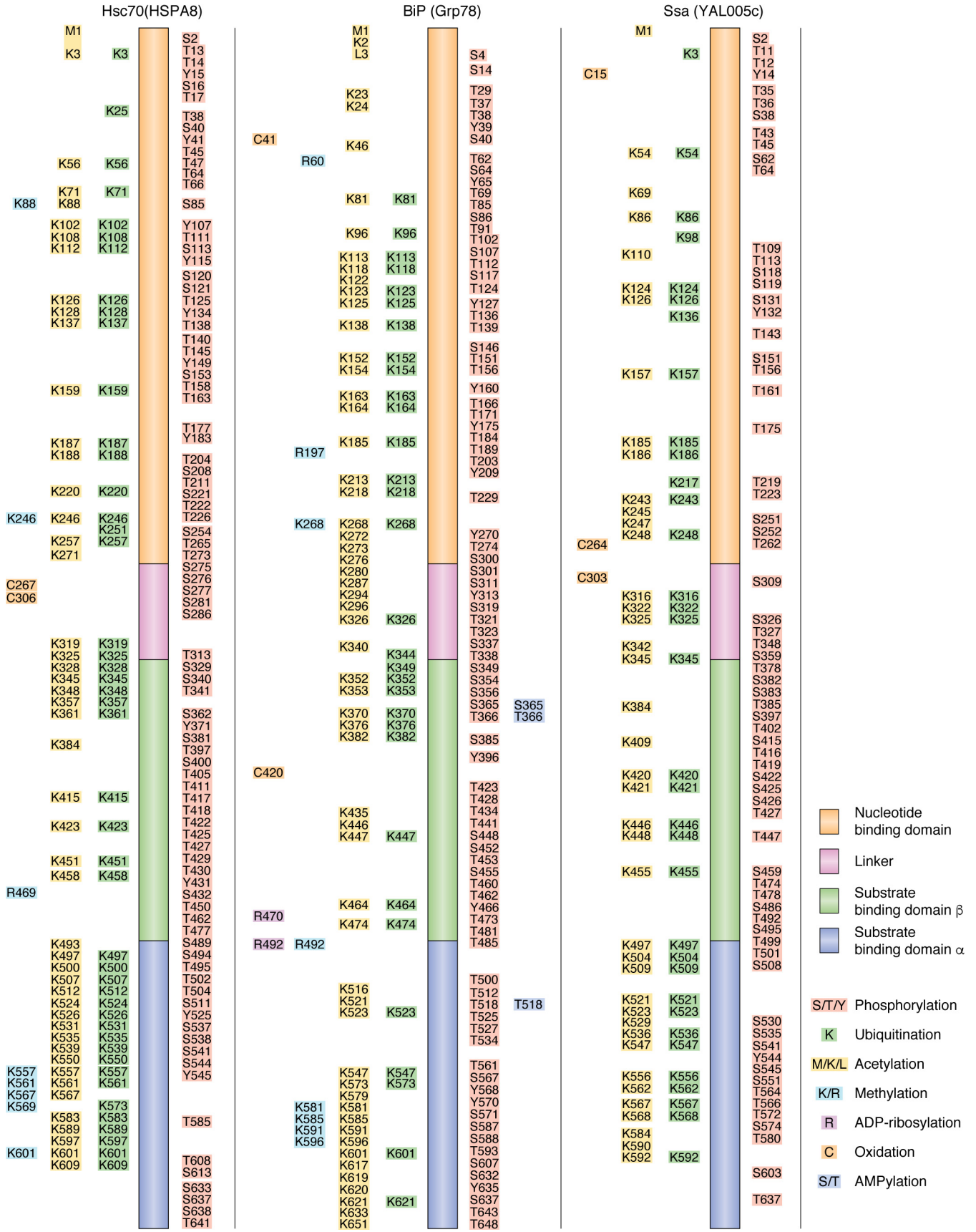

Figure 1. The post-translational modifications of mammalian Hsp70, Hsc70, and BiP and yeast Ssa1–4. Shown is a domain representation of the Hsp70 family members, with detected PTMs marked with appropriate residue numbers. PTMs are labeled as follows: phosphorylation in *red*, ubiquitination in *green*, acetylation in *yellow*, methylation in *cyan*, ADP-ribosylation in *purple*, and AMPylation in *dark blue*.

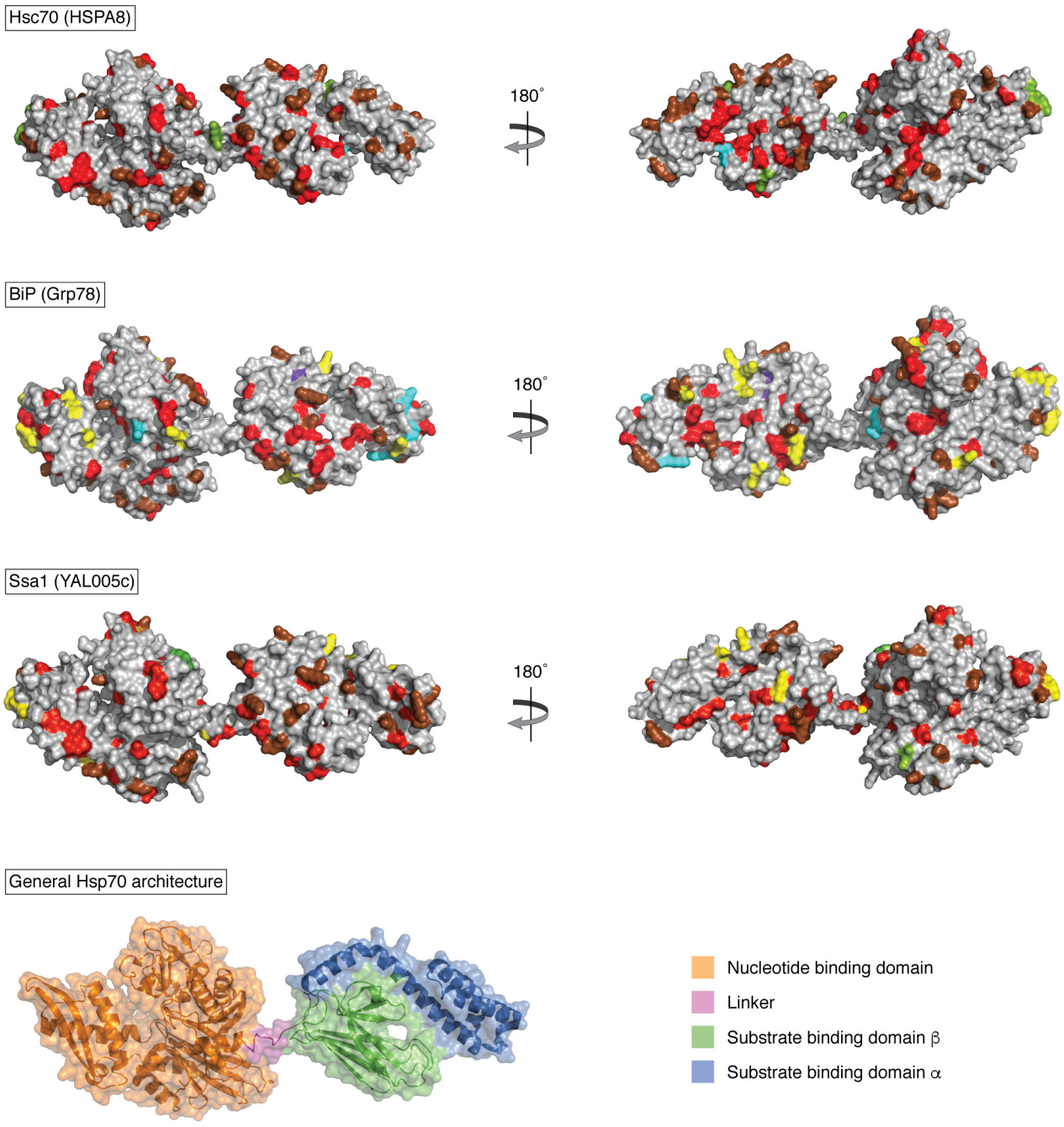

Figure 2. Locations of PTMs on the Hsp70 structure. PTMs were mapped onto predicted structural models created by SWISS-MODEL based on Protein Data Bank entry 2KHO for each Hsp70 isoform (192). PTMs are *colored* as in Fig. 1, except sites of multiple modification are *labeled brown*.

reveals an astonishing number of PTMs on Hsp70s (Fig. 1). Below, we summarize the current state of knowledge of Hsp70 PTMs, including phosphorylation, acetylation, ubiquitination, AMPylation, ADP-ribosylation, and methylation, and their conservation and potential interplay.

Hsp70 phosphorylation

Over 100,000 phosphorylation events occur in mammalian cells, and improper protein phosphorylation is the cause of many human pathologies (61). Hsp70 family proteins are highly phosphorylated; a total of 88, 87, and 70 phosphorylated sites have been identified so far on Hsc70, BiP, and yeast Ssa1, respectively (Figs. 1 and 2). Nevertheless, a mechanistic understanding of how individual phosphorylation events impact distinct Hsp70 functions remains elusive, with exceptions discussed below.

Hsp70 phosphorylation in cell cycle progression and cell proliferation

Cells must tightly coordinate growth and division in response to a variety of internal and external cues that include nutrient availability and genome integrity. These signals are sensed and propagated throughout cells via complex interlinked signal transduction pathways. Early studies on the cell

cycle had demonstrated a role for the yeast co-chaperone Ydj1 in regulating the entry of the Cln3 G_1 cyclin into the nucleus (62). Cln3 possesses a J-domain–like region and competes with Ydj1 for binding to Ssa1. In 2012, Truman *et al.* (56) established that the molecular trigger for displacement of Ydj1 and recruitment of Cln3 was phosphorylation of Ssa1 on Thr-36 mediated by two related CDKs (Pho85 and Cdk1). These two kinases activate Thr-36 under different cellular conditions. During periods of nutrient scarcity, Pho85 phosphorylates Ssa1, promoting Cln3 recruitment to Ssa1 and its eventual destruction, leading to G_1 cell cycle arrest. In cycling cells, transient Thr-36 phosphorylation of Ssa1 by Cdk1 promotes Cln3 destruction at the end of G_2/M, resetting G_1 cyclin levels in preparation for the beginning of the next cell cycle. Suggesting an evolutionarily conserved mechanism, phosphorylation of the equivalent Thr-38 residue on mammalian Hsc70 promotes Hsc70-cyclin D1 interaction and cyclin D1 destruction.

Although clearly important for G_1/S progression, Hsp70 phosphorylation can also impact later stages of the cell cycle. Polo-like kinase 1 (Plk1) is another serine/threonine kinase that regulates mitotic entry to cytokinesis and cell cycle progression (63). In arsenic trioxide–treated mitotically arrested HeLa S3 cells, Plk1 phosphorylates Hsp70 at five sites: Thr-13, Thr-226, Ser-326, Ser-631, and Ser-633. The phosphomimetic mutants of Ser-631 and Ser-633 increase the proportion of cells arrested at mitosis. Thus, Plk1-mediated phosphorylation of Hsp70 plays a protective role against cell death by apoptosis. However, the significance of the other three phosphorylation sites by Plk1 remains obscure, and mechanisms by which Hsp70 provides protection remain to be explored. Hsp70 phosphorylated at Ser-631 and Ser-633 co-localizes with Plk1 at the centrosome. This association leads to an increase in microtubule stability, elongation of mitotic spindles, and mitotic arrest. Furthermore, when the interaction of Hsp70 and Plk1 is inhibited by 2-phenylethynesulfonamide or by inhibiting Plk1 activity by using BI2536, the apoptotic inhibition is released and leads to cell death. Hence, the phosphorylation of Hsp70 at Ser-631 and Ser-633 promotes Hsp70's role as a centrosomal chaperone (64). Although the mechanism of this apoptotic release still remains unclear, the inhibition of Hsp70 phosphorylation or Plk1 inhibition might serve as a key to increase efficiency of arsenic trioxide as a chemotherapeutic drug. In addition to Plk1, the mitotic Nek6 kinase phosphorylates Hsp72 in the NBD at Thr-66, promoting recruitment of Hsp72 to the mitotic spindles (65). This phosphorylation event activates the alignment of chromosomes to the spindle equator by stabilizing kinetochore fibers. This is achieved via recruitment of two proteins, ch-TOG and TACC3, to kinetochore fibers. Phosphoinhibitory mutants of Hsp72 fail to localize at spindle poles, resulting in the destabilization of kinetochore fibers, leading to abnormal mitotic progression (65).

Whereas it has been known for several years that chaperones can bind and regulate MAP kinase function (66), a recent study suggests that mitogen-activated protein kinase pathway activity can in turn regulate chaperone function (67). Treatment of cells with epidermal growth factor triggers Hsp70 phosphorylation on Ser-385 and Ser-400. These sites are particularly interesting as they reside on and adjacent to the flexible linker that connects the NBD and SBD of Hsp70. Phosphorylation of Hsp70 at these two sites leads to an extended conformation of the protein, which in turn increases the binding affinity of the clients (67). Mutation of these two sites results in cells that have a reduced viability and growth rate. Although clearly dependent on the activity of extracellular signal–regulated kinase (ERK), these two phosphorylation sites do not fulfill the minimum requirements for ERK phosphorylation (proline in the +1 position to the site of phosphorylation) and thus are highly unlikely to be direct ERK substrates (68–70). Interestingly, Ser-400 lies next to a putative NLS on the Hsp70 sequence, and the S400A mutation prevents its nuclear export (71). Going forward, it will be interesting to identify the specific kinase for these sites, the impact these sites have on co-chaperone binding, and the specific clients impacted that result in the increased cellular proliferation. Taken together, it is clear that several sites on Hsp70 and Hsc70 are regulated by multiple kinases throughout the cell cycle, in turn leading to the targeted stabilization/destabilization of key effectors of the cell cycle.

Hsp70 phosphorylation in cancer

Many of the driver mutations involved in cancer alter writer and eraser enzymes in signal transduction pathways (72). It is a likely conclusion that chaperone PTMs will be altered to varying degrees in different cancers. These changes may impact the stability of oncoproteins, activity of key pathways required for tumorigenesis, and anticancer drug resistance. A known example of this is alteration of methotrexate resistance in leukemia cells upon Hsc70 tyrosine phosphorylation. The interaction between Hsc70 and reduced folate carrier protein (RFC), the primary transporter of folate and antifolate drugs, regulates cellular methotrexate uptake (73). Tyr-288 phosphorylation is required for the binding of Hsc70 to methotrexate. A phosphoinhibitory mutant of Tyr-288 disrupted the interaction between Hsc70-RFC and methotrexate, which affects its transport into the cells, rendering the cells resistant to this drug (35). Decreases in tyrosine phosphorylation of Hsc70 further lead to increased methotrexate resistance in these cells (74).

Hsp70 phosphorylation exerts anti-apoptotic properties upon serum starvation in hepatocellular carcinoma. Retinoic acid–induced 16 (RAI16) is a protein kinase A–anchoring protein, which gets activated in response to serum starvation and drives protein kinase A–mediated phosphorylation of Ser-486 on Hsp70, thus preventing caspase-3 cleavage and apoptosis (75). Further studies are required to identify the key players that mediate these phosphorylation and dephosphorylation events so that the properties of PTM-based Hsp70 and Hsc70 regulation in cancer can be utilized as a novel chemotherapeutic option.

Hsp70 phosphorylation as regulator of Hsp70 client triaging

Although Hsp70 is required for the folding of both new synthesized and misfolded proteins, it is also able to target damaged proteins for degradation via the ubiquitin-mediated proteasomal system (76). The strategy that Hsp70 uses to decide whether to fold or degrade a client still remains unclear.

The protein-folding and degradation activities of Hsp70 are mediated by its co-chaperones Hop and CHIP, respectively. The co-chaperone binding to C-terminal of Hsp70 is facilitated by the interaction between the tetratricopeptide repeat region of both Hop and CHIP with the C-terminal domain of Hsp70 (77). Phosphorylation of Hsp70 at Thr-636 leads to an increase in Hsp70-HOP binding and a corresponding decrease in Hsp70-CHIP binding, leading to increased client stability. Loss of this phosphorylation has the converse effect, triggering enhanced client degradation resulting from Hsp70-CHIP interaction (58). Similarly, another study demonstrated that the SBD of Hsp70 is phosphorylated by Akt1 at Ser-631, which decreases Hsp70-CHIP interaction (78). In this report, the downstream effect observed was to prevent degradation of superoxide dismutase-2 (SOD2) and promote the import of SOD2 into the mitochondria. In this way, Hsp70 regulates SOD2 activity and is a dynamic regulator of mitochondrial redox homeostasis.

Hsp70 phosphorylation in host-pathogen interactions

Hsp70 folds a large proportion of cellular proteins and thus supports the activity of a wide range of signaling pathways. It is thus unsurprising that Hsp70 phosphorylation appears to be altered in both hosts and pathogens in response to infection. *Legionella pneumophila* secretes over 300 bacterial effector proteins into target host cells, several of which are eukaryotic-like Ser/Thr protein kinases (79). One of these kinases, LegK4, phosphorylates the host cytosolic Hsp70 and Hsc70 at Thr-495 in the SBD. Phosphorylation of this well-conserved site reduces the ability of the HDJ1 co-chaperone to stimulate the ATPase activity of Hsc70. At a cellular level, the knock-on effect of this phosphorylation is a reduction in the unfolded protein response and protein synthesis. Overall, this study reveals a fascinating mechanism by which *Legionella* is able to directly manipulate chaperone function to increase its probability of survival in host cells.

Hsp70 phosphorylation in pathogens themselves may also be important for infectivity. In the opportunistic pathogenic yeast *Candida albicans*, Ser-361, Tyr-370, and Thr-576 are constitutively phosphorylated. Upon transition to the pathogenic hyphal form, an additional eight sites (Thr-11, Thr-136, Thr-161, Thr-175, Ser-328, Thr-387, Thr-494, and Ser-578) become activated (80). Mutation of these sites produces cells with growth defects, including loss of the ability to form hyphae.

In a similar manner, the malarial parasite *Plasmodium berghei* displays increased phosphorylation of Hsp70 at the gametocyte stage. This phosphorylation occurs on Ser-106, Ser-585, Thr-587, and Ser-588 (81). Although more mechanistic studies are required, these phosphorylation sites may have a possible role in conferring protection to the parasite in its host. It is interesting to note that some of the activated sites seen in host-pathogen interactions are conserved in other organisms under differing conditions. The functional changes brought about by these phosphorylations may have evolved to be utilized in organism-specific manners.

BiP phosphorylation

Early studies identified BiP as being serine/threonine-phosphorylated and that this may represent an inactive, noncomplexed form of BiP (82). Follow-up studies revealed that BiP phosphorylation is associated with a dimeric form and that its phosphorylation decreases upon cellular exposure to ER stressors such as tunicamycin (83, 84). Taken together, the current model suggests that phosphorylation may promote a dimeric, inactive form of BiP. Upon ER stress, BiP phosphorylation decreases, allowing dimer dissociation and formation of BiP-client complexes. Whereas many sites of phosphorylation have been detected on BiP (Figs. 1–3), their individual functions remain unclear.

Hsp70 AMPylation (adenylylation, adenylation)

AMPylation (also known as adenylylation and adenylation) involves the covalent addition of AMP to serine, tyrosine, or threonine residues (85). Enzymes responsible for AMPylation (AMPylases) have been identified in all three domains of life (86). Characterized AMPylases belong to four groups: Fic (filamentation induced by cAMP) domain-containing proteins (87), GS-ATase (glutamine synthetase adenylyltransferase), SelO (88), and DrrA (*Legionella pneumophila* effector protein) (89). Fic-domain–containing proteins are found in Archaea, Bacteria, and Eukarya, whereas SelO is found in Bacteria and Eukarya. GS-ATase is only present in Bacteria, and DrrA is restricted to strains of *Legionella pneumophila* (86, 90). Most metazoans express a single fic type AMPylase, all of which share a highly conserved catalytic site architecture characterized by the fic motif H*X*F*X*(D/E)GNGR*XX*R (16, 91). Metazoan AMPylases and their functions, particularly their roles in the regulation of Hsp70 family chaperones, have gathered increasing attention in recent years. AMPylation is considered to inhibit the activity of Hsp70 family chaperones, conserving a pool of chaperones in a "stand-by," or "primed," state, which can rapidly respond to stress when de-AMPylated (92, 93). The AMPylase FICD functions as a bidirectional enzyme, catalyzing both AMPylation and de-AMPylation involving a single active site (93, 94). This bidirectionality is regulated by the oligomeric state of FICD, with monomeric FICD acting as an AMPylator and the dimeric form de-AMPylating (92, 95).

AMPylation of Hsp70s has been investigated in *Homo sapiens* (humans), *Cricetulus griseus* (Chinese hamsters), *Drosophila melanogaster* (fruit flies), and *Caenorhabditis elegans* (worms). In each of these species, at least one member of the Hsp70 family is regulated by AMPylation. In Chinese hamster ovary (CHO-K1) cells, FICD AMPylates the ER-resident Hsp70 BiP on Thr-518 (96). AMPylation levels increase and decrease inversely to unfolded protein burden (96). When AMPylated, BiP's substrate "on" and "off" rate is elevated, basal ATPase activity is decreased, and J-protein co-chaperone–stimulated ATP hydrolysis activity is attenuated, demonstrating that AMPylation is an inactivating modification in CHO-K1 cells (96). However, BiP AMPylation does not prevent the binding of ATP to BiP's active site nor the recruitment of J-type co-chaperones. AMPylated BiP is thus proposed to be trapped in an ATP- and J-domain co-chaperone–bound conformation that

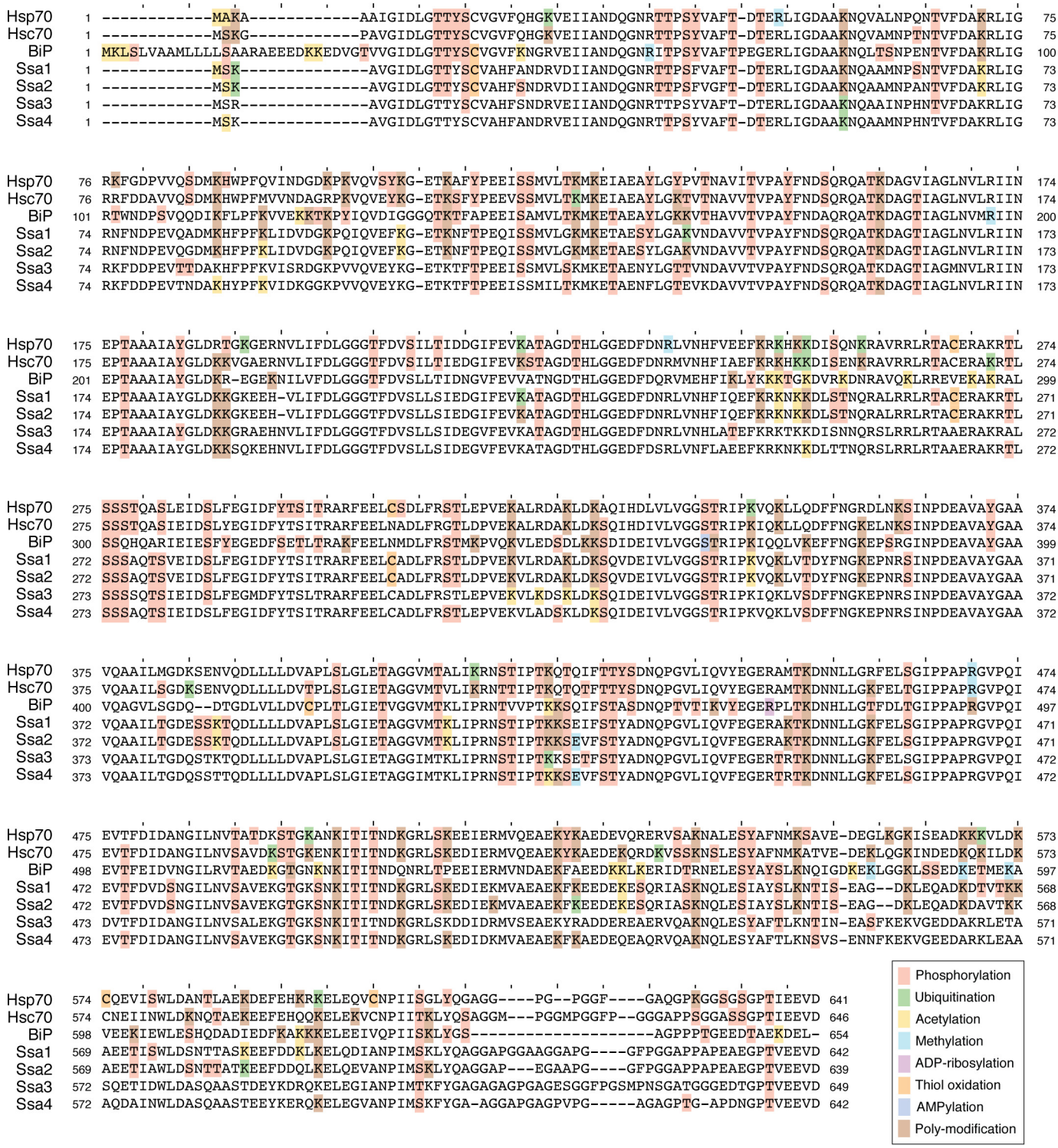

Figure 3. Conservation of Hsp70 PTMs and surrounding sequence between Hsp70 isoforms. Alignment was build using human Hsp70, Hsc70, and BiP as well as *S. cerevisiae* Ssa1, Ssa2, Ssa3, and Ssa4 sequences. Sequences were aligned using ClustalX (193), and sites of PTM were labeled as in Fig. 2.

will engage in protein-refolding activities immediately following its de-AMPylation (96). Even when BiP is AMPylated in its ADP-bound state, it adopts an ATP-like state, with impaired oligomerization (97). Increasing BiP AMPylation by the expression of FICD (E234G), the constitutively AMPylating mutant of FICD, induces the UPR^{ER} in CHO-K1 cells, whereas FICD-inactivated cells show a delayed UPR^{ER} in rat pancreatic acinar AR42j cells, suggesting a regulatory role for BiP AMPylation in UPR^{ER} induction (96). WT FICD, but not the E234G or the non-AMPylating H363A FICD mutants, is able to reverse AMPylation *in vitro*. In fact, when WT FICD is co-expressed with FICD (E234G) in CHO-K1 cells, the AMPylation levels and UPR effects of FICD (E234G) are mitigated (93, 94).

The *Drosophila* ortholog of BiP is AMPylated and deAMPylated by dFic at Thr-366 and Thr-518 (92, 98). AMPylation occurs in a Ca^{2+}-dependent manner, and preferentially on the inactive conformation of BiP (98). During homeostasis, BiP is highly AMPylated, whereas AMPylated BiP levels decrease when unfolded proteins accumulate in the ER, suggesting that AMPylation also plays an inactivating role in *Drosophila*. Further, transcription of both BiP and dFic is increased in response to ER stress (98). dFic can also be secreted and locate to the surface of capitate projections (glial projections that insert into the axons of photoreceptors in the eye of flies), and loss of dFic leads to blindness in flies, due to loss of postsynaptic neuron activation (98). BiP(T366A) is resistant to AMPylation, and BiP (T366A) flies also exhibit loss of synaptic function, indicating that the effects of dFic loss are caused by deregulation of BiP (99). Expression of a constitutively active dFic mutant (FicE247G) in a dFic knockout background is lethal but tolerated in the presence of WT dFic or BiP(T366A), further demonstrating the importance of residue Thr-366 in AMPylation-mediated BiP regulation in *Drosophila* (98, 100).

In *C. elegans*, the BiP/GRP78 orthologs HSP3 and HSP4 and the cytosolic Hsc70 ortholog HSP1 are AMPylated (101, 102). Illustrating the role of AMPylation in regulation of Hsp70s, in strains expressing aggregating amyloid β or α-synuclein, hyper-AMPylation increases the formation of large protein aggregates while decreasing their cytotoxicity (103–106). RNAi-mediated knockdown of Hsp70 family chaperones HSP1 as well as HSP3 and HSP4 phenocopies this reduction in aggregate toxicity observed in strains with increased AMPylation levels, suggesting that the effects of hyper-AMPylation are caused by AMPylation-mediated inactivation of Hsp70s in these worms (105). The site of AMPylation on HSP3 is Thr-176, but the AMPylation site(s) of HSP-1 and HSP-4 remain to be mapped (105). Expression of the constitutively active *C. elegans* AMPylase Fic-1(E274G) in *S. cerevisiae* induces up-regulation of the heat shock response and HSPs expression, as well as growth arrest and increased protein aggregation (101). These phenotypes are partially rescued by increasing the expression of the cytosolic Hsp70 family member Ssa2, suggesting that Fic-1(E274G) targets Hsp70 family proteins in budding yeast as well (101).

Human BiP is AMPylated at Thr-366 and Thr-518 (94, 107). These sites play slightly different roles in regulation of the Hsp70 ATPase cycle. Whereas AMPylation of Thr-366 increases basal ATPase activity with no effect on J-protein–stimulated ATPase activity, AMPylation of Thr-518 decreases J-protein–stimulated ATPase activity with no effect on basal ATPase activity (94, 107). In addition to BiP, human FICD also AMPylates other major chaperones, including Hsp70, Hsp40, and Hsp90 (101). *In vitro*, Hsp70 is AMPylated on five threonine residues in the ATPase domain (101). In HEK293T cells, expression of FICD(E234G) induces higher expression of BiP, presumably to compensate for the BiP inactivated by AMPylation (96). Transfection of HeLa cells with the constitutively AMPylating FICD(E234G) leads to up-regulation of the heat shock response and activation of heat shock factor 1 (HSF1), a key transcription factor negatively regulated by Hsp70 that promotes the transcription of chaperones and thereby regulates the heat shock response (101, 108). FICD(E234G) also inhibits translocation of Hsp70 to the nucleus (101).

A recent screen for AMPylated proteins in eight different human cell lines using a novel N6pA probe further revealed that HSPA2, HSPA4, BiP, Hsc70, and mortalin are AMPylated by endogenous FICD in a cell type–specific pattern (109): whereas BiP was modified in all cell lines, HSPA2 (fibroblasts), HSPA4 (fibroblasts), Hsc70 (fibroblasts, SH-SY5Ys), and HSPA9 (fibroblasts, IPSCs) were AMPylated in a subset of the tested cell types. This supports the claim that AMPylation elicits regulatory functions on distinct Hsp70 family members.

Taken as a whole, these studies show that AMPylation occurs on threonine and serine residues of several Hsp70 family proteins and is particularly important in regulating BiP function. Whether BiP is simultaneously AMPylated on multiple residues or Thr-365 and Thr-518 are uniquely modified in response to distinct cues remains to be defined. The majority of studies have shown that this modification inactivates or inhibits the activity of Hsp70 proteins, at least with regard to its chaperone activities. This suggests a model in which AMPylation acts as a "holding" mechanism, in which a steady level of Hsp70s can be maintained in the cell inactive during times of low stress, but ready to rapidly respond to stress events without the need to wait for transcription and translation. The homeostasis/stress AMPylation cycle has not yet been worked out for all Hsp70 family members. In the case of BiP, however, under basal nonstressed conditions, BiP appears to be largely AMPylated, and when stress is induced, BiP is de-AMPylated in response. AMPylation can have both advantageous and deleterious effects on cells, depending on context. Whereas constitutive AMPylation leads to activation of the UPR in cell models, no AMPylation leads to delayed UPR activation (93, 96). In flies, constitutive AMPylation is lethal, but lack of AMPylation leads to blindness (99, 100). In *C. elegans* protein aggregation models, increased AMPylation leads to increased protein aggregates but reduces the toxicity of those aggregates (105). This balance reflects the tight balance needed to maintain homeostasis and illustrates the crucial role of AMPylation.

Hsp70 ADP-ribosylation

Another post translational modification observed on Hsp70s is ADP-ribosylation (82, 110–115). ADP-ribosylation, a process catalyzed by ADP-ribosyltransferases (ARTs), involves the transfer of an ADP-ribose from NADH (NAD^+) onto a target protein with a nucleophilic oxygen, nitrogen, or sulfur (110, 111). ARTs fall into three families of proteins: arginine-specific ADP-ribosyltransferases (ART/ARTC), poly(ADP-ribose) polymerases (PARP/ARTD), and sirtuins. Proteins can be either mono- or poly-ADP-ribosylated (110, 111). ADP-ribosylation can be removed by hydrolysis by ADP-ribosyl hydrolases or hydrolases of the macrodomain family (116). In CHO and human HEK293T and HeLa cells, BiP is ADP-ribosylated by hamster ARTC2.1 and by human ART1, respectively.

Early studies identified several conditions that induced BiP ADP-ribosylation, including suspension of protein translation and ER stress (114, 117–119). In mice, BiP is less ADP-ribosylated in B cells making γ1-heavy chains than in those making

neither heavy nor light chains (82). In quiescent Swiss 3T3, Rat-1 cells, and mouse embryonic fibroblasts, BiP is ADP-ribosylated; however, when proliferation is induced, ADP-ribosylation is reduced (120). When mice are fasted overnight or administered cycloheximide to halt protein production, their pancreases have increased levels of ADP-ribosylated BiP, which is reversed by feeding (119).

The specific sites and exact function of ADP-ribosylation have not been well-validated. MS-based studies have identified ADP-ribosylation at residues Asp-78 and Lys-81 of BiP, as well as Asp-53 of Hsc70 in HeLa cells, and residue Arg-50 of BiP and Arg-346 of HSPA13 in murine skeletal muscle (121). Additionally, mutation of either Arg-470 or Arg-492 to lysine in hamster BiP substantially decreases ADP-ribosylation, and whereas ribosylation-mimicking mutations decrease client binding, they do not appear to alter intrinsic ATPase activity. It should be noted that the authors did not definitively identify Arg-470 or Arg-492 as a site of ribosylation (by MS or other related technologies), and advances in the field suggest that these sites may also be modified by AMPylation (see below). On size-exclusion chromatography columns, ADP-ribosylated BiP is present in lower-molecular weight fractions, indicating that ADP-ribosylation prevents BiP participating in multichaperone complexes (119).

Additionally, ADP-ribosylation is only found on the oligomeric form of BiP, which is the predominant form under low protein-folding burden (83). Combined, these studies indicate that higher levels of ADP-ribosylated BiP are found during low protein production/low unprocessed protein burden (82, 114, 117–119). On the other hand, BiP ADP-ribosylation is decreased when the unprocessed protein burden is higher, as when more proteins are being produced and when protein glycosylation is hindered (82, 120, 122). Taken together, this suggests that ADP-ribosylation may play a role in regulation of BiP's chaperoning activities.

Overall, studies on BiP ADP-ribosylation describe a modification that is most present under conditions in which BiP's folding activities are not needed and which may be inhibitory to BiP's chaperoning functions. Inversely, when unfolded protein burden is high, or when BiP is actively bound to a client protein, ADP-ribosylation levels are low. This suggests that ADP-ribosylation may act as a temporary "off" switch for BiP.

Studies on ADP-ribosylation and AMPylation have been complicated by the fact that early studies on ADP-ribosylation used radiolabeled adenosine, which can also label AMPylated sites. Cleavage by an ADP-ribosyl hydrolase was not used to confirm ADP-ribosylation in the above studies. Chambers *et al.* (119), investigating BiP arginine modification, point out that the identified mammalian arginine hydrolase, ARH1 (116), is a cytoplasmic protein and unlikely to act on BiP. In some of these studies, ADP-ribosylation was confirmed by digestion with snake venom phosphodiesterase, which cleaves phosphodiester bonds (123), and by blocking ADP-ribosylation using nicotinamide, which inhibits ARTs as well as PARPs (124). Importantly, snake venom phosphodiesterase is also able to cleave the phosphodiester bond of an AMPylation modification. Furthermore, nicotinamide treatment has many effects on the cell other than blockage of ADP-ribosylation, including stimulation of DNA damage repair and blocking hexose uptake, which could in turn have effects on cellular metabolism leading to decreases in AMPylation, not ADP-ribosylation (125, 126). In fact, Preissler *et al.* (96), the same group that published Chambers *et al.* (119), themselves postulate that previous studies of BiP ADP-ribosylation may have been mistakenly characterizing BiP AMPylation (96).

Hsp70 acetylation

Acetylation involves the transfer of an acetyl group onto a lysine residue, the source of which is the metabolite acetyl-CoA (127). Acetylation is catalyzed by three related families of lysine acetyltransferases, GCN5, p300, and MYST, whereas removal of acetyl groups is processed by Sirtuin NAD-dependent lysine deacetylases (127). The Hsp70 family of proteins are heavily modified by acetylation; a total of 50, 58, and 40 acetylated sites have been identified so far on Hsc70, BiP, and yeast Ssa1, respectively (Figs. 1–3). As with phosphorylation, acetylation fine-tunes Hsp70 function in a wide range of cellular processes.

Hsp70 acetylation control of the heat shock response

The historical paradigm for the heat shock response is that during heat shock, Hsp70 levels are induced through increased expression to compensate for the increase in unfolded proteins (62, 128). A recent study in yeast has revealed that in addition, Ssa1 is rapidly deacetylated at four lysine residues, Lys-86, Lys-185, Lys-354, and Lys-562, in response to heat shock (129). These deacetylation events are required for interaction with key co-chaperones such as Ydj1, Zuo1, Sgt2, and Hsp26 during heat shock. Fascinatingly, the inducible Hsp70s (Ssa3 and Ssa4) have an alanine at position 562 and thus cannot undergo acetylation at this site. It is interesting to speculate that this naturally occurring mutation in Ssa3 and Ssa4 makes them immediately prepared for action when expressed during heat shock (for more on isoform differences in the code, please see below).

Hsp70 acetylation in client triaging and cell survival

In response to oxidative stress, the ARD1 acetyltransferase acetylates Hsp70 at Lys-77, allowing Hsp70 to bind to Hop and allowing refolding of denatured clients. After longer periods of stress, Hsp70 becomes deacetylated, promoting interaction with CHIP to degrade damaged proteins. This switch from protein refolding to degradation is required for the maintenance of protein homoeostasis and protects the cells from stress-induced cell death. Thus, in a similar manner to phosphorylation-mediated triaging reported by Muller *et al.* (58), ARD1-mediated Hsp70 acetylation is a regulatory mechanism that balances protein refolding/degradation in response to stress (130). In addition to altered co-chaperone binding, Hsp70 acetylation on Lys-77 facilitates its binding with pro-apoptotic proteins Apaf-1 and AIF and inhibits Apaf-1– and AIF–dependent apoptosis. Hsp70 acetylation also attenuates autophagy by Atg12-Atg5 complex formation, Beclin-1 expression, and perinuclear LC3 puncta formation, resulting in the inhibition of autophagic cell death. It is worth noting that only the inducible Hsp70 has Lys-77; the corresponding site in the

constitutive Hsc70 is a nonacetylatable arginine, suggesting isoform-specific functionality.

Aside from Lys-77, other Hsp70 acetylation sites can impact autophagy. Post-amino acid starvation, Hsp70 Lys-159 acetylation is up-regulated. Acetylated Hsp70 displays enhanced binding affinity to KAP1 (SUMO E3 ligase), which in turn increases the SUMOylation of autophagy protein Vps34. This newly formed Hsp70-KAP1-Vps34 complex binds to Beclin 1 (complexed to autophagy proteins ATG14L, Ambra1, Bif1, or UVRAG) and promotes phagophore formation. Subsequently, the ULK kinase complex (ULK1/2, ATG13, FIP200) proteins are recruited to the phagophore. Finally, the membrane encloses the cytosolic cargos, resulting in the formation of an autophagosome. Thus, under nutrient starvation, acetylation of Hsp70 is a key step for activating autophagy (131). Interestingly, in contrast to Hsp70 Lys-77 acetylation, Lys-126 acetylation weakens its binding to Hop and Hip yet strengthens the interaction between CHIP and Bag1. Lys-126 acetylation also inhibits Hsp70-mediated tumor cell invasion and migration and the binding of Hsp70 to AIF1 and Apaf1 for promoting mitochondria-mediated apoptosis (132). Going forward, it will be interesting to underpin how activation and function of Lys-77, Lys-126, and Lys-159 acetylation relate to one another.

Hsp70 methylation

Methylation represents a highly abundant PTM found on all Hsp70 isoforms (133–135). This modification is conferred by three enzyme families: methyltransferase-like proteins (METTL) (136, 137), protein arginine methyltransferases (PRMTs) (133, 135), and SET domain–containing histone methyltransferases (SETD) (138). Modifications occur on surface-exposed lysine residues, which can be mono- (me1), di- (me2), or trimethylated (me3) (139), and arginine residues, which are monomethylated, asymmetrically dimethylated, or symmetrically dimethylated (139, 140). Most sites of methylation were found in proteome-wide MS studies (134, 135, 137, 141). The functional consequences of Hsp70 methylation, with a few exceptions discussed below, remain poorly understood.

Hsp70 methylation in the regulation of gene expression

Histone and DNA methylation are well-known regulatory traits that control gene transcription. Recent advances in our understanding of histone methylases and demethylases highlight that some of these enzymes are also capable of modifying nonhistone substrates, including Hsp70 family chaperones. Upon methylation of the conserved Arg-469 residue, Hsp70s associate with chromatin and regulate retinoid acid–dependent retinoid acid receptor β2 (RARβ2) gene expression (133). Whereas both the methylation-incompetent R469A mutant and WT Hsp70 bind to the promoter region of RARβ2, only methylated Hsp70 recruits TFIIH to the preinitiation complex during RARβ2 transcription initiation (133). Arg-469 methylation is conferred by PRMT4, which monomethylates the conserved Arg-469 residue across Hsp70 isoforms (133). This methylation is at least partly removed from Hsp70s by the JmjC-domain–containing demethylase JMJD6 (133). Together, PRMT4 and JMJD6 constitute a classic "writer-eraser" pair that regulates transcriptional events through Hsp70, rather than histone methylation.

Hsp70 methylation in direct regulation of chaperoning function

The protein-folding and -refolding activities of Hsp70 chaperones are in part regulated by lysine methylation events. Hsc70 Lys-561 and the orthogonal residues in BiP, Hsp70, HSPA2/Hsp70-2, and HSPA6/Hsp70B′ are trimethylated by the nonhistone methylase METTL21A (134, 136, 137, 142). In the presence of ER stress, BiP K586me3 is degraded by the lysosome and replaced with methyl-free *de novo* translated BiP, implicating a role for K586me3 in the regulation of BiP's chaperoning function (143). Hsc70 Lys-561 trimethylation affects its ability to bind to substrates, such as α-synuclein (136). Conversely to BiP, methylation-mimetic Hsc70 K561R is more stable than a K561A nonmethylatable protein, suggesting that Hsc70 K561me3 regulates Hsc70 turnover and degradation (144). There are several possible explanations for the apparent opposite effects of trimethylation on BiP and Hsc70. One is that they are separate proteins in separate subcellular compartments, and so this same modification may play different roles in specific contexts. Additionally, BiP K586me3 is degraded following ER stress, as are several other ER-resident chaperones (143). Finally, the stabilizing effects of Hsc70 K561me3 were studied using a lysine-to-arginine mutation to mimic lysine methylation (144). However, another group used a Hsp70 K561R mutant as nonmethylatable mutant (138), illustrating the complications of interpreting mutants. In addition, as others have pointed out, this is a conserved residue, and so mutants may investigate the importance of an important residue rather than methylation (142). A possible mechanism was proposed by Zhang *et al.* (145), showing that Hsc70 Lys-561 trimethylation interferes with CHIP-mediated Hsc70 ubiquitination (144). Interestingly, Lys-561 methylation is not required for Hsc70-dependent chaperone-mediated autophagy; nor is Lys-561 trimethylation inhibitory to Hsc70's chaperoning function (144). Knockout of METTL21C, a close paralogue of METTL21A, leads to increased Hsc70 Lys-561 dimethylation, indicating possible competing roles of di- and trimethylators (144). *In vitro*, METTL21A predominantly catalyzes mono- and dimethylation of Hsp70, BiP and Hsc70, with trimethylation only occurring at high METTL21A concentrations (136). METTL21A knock-out cells are deficient in Hsp70 methylation, suggesting that this enzyme is strictly required for Hsp70 Lys-561 methylation (142). Localization studies are conflicting. Cho *et al.* (138) found Hsp70 K561me2 predominantly located in the nucleus of cancer cells, whereas all other HSP70 predominantly localized to the cytosol. In contrast, Jakobsson *et al.* (142) found me0, 1, 2, and 3 HSP70 Lys-561 in both the cytosol and nuclei in HeLa and HEK293 cells and found HSP70 K561me3 to be the most prevalent form in either compartment, with the relative amounts of each methylation status the same between both compartments. Gao *et al.* (133) also found Hsp70 R469me1 in both cytosolic and nuclear compartments. Cloutier *et al.* (137) found that most METTL21A and HSC70 in cells overexpressing METTL21A localizes to the cytosol. Further

studies are needed to clarify how methylation of different Hsp70s affects localization, and which additional methylases might be critically involved in Hsp70 Lys-561 modification remains to be defined in detail (134, 136, 137, 142).

Given that methylated Hsp70-R469me1 promotes RARβ2 transcription (133) and is more stable (144), whereas Hsc70 K561me3 has impaired substrate-binding abilities (136), it is likely that methylated and nonmethylated Hsp70s play different physiologic roles. Just as Hsc70 K561me3 has reduced substrate affinity (136), this may be true for other trimethylated Hsp70s. As such, trimethylated forms of these chaperones would be expected to be lower in the case of high unfolded protein burden. Meanwhile, other functions of Hsp70s, such as induction of target protein degradation (144) and transcript initiation (133), appear to rely on trimethylation of Hsp70s. These diverse roles might occur in different cell compartments, which would explain why some methylated Hsp70 forms and methylators are restricted to or enriched in specific cellular compartments.

Hsp70 family methylation has been identified in other organisms, yet at a lower frequency. In yeast, Hsp70 isoforms Ssa2 and Ssa4 are monomethylated on Lys-421/422 or Glu-423/424, respectively, the consequence of which remains unknown (146).

Ubiquitination

In contrast to small chemical modifications on amino acids, ubiquitination refers to the covalent addition of an 8-kDa ubiquitin (Ub) protein to substrate proteins. Ubiquitination is an ATP-dependent process and is catalyzed by the sequential activity of three enzymes. First, ubiquitin-activating enzymes (E1) adenylate Ub and load this primed Ub unit to one of ∼40 ubiquitin-conjugating enzymes (E2). The Ub-containing E2 enzyme forms a complex with one of ∼600 ubiquitin ligases (E3) (147, 148). Directed by the E3 ligase, the E2-E3 complex finally interacts with its substrate and promotes the formation of an isopeptide bond connecting the carboxyl-terminal glycine residue of Ub with an accessible lysine residue. The presence of seven lysines in Ub itself (Lys-6, -11, -27, -29, -33, -48, and -63) enables the assembly of branched poly-Ub chains, which have different implications for substrate fates (149). Whereas Lys-48–linked Ub chains label substrates for degradation by the 26S proteasome, Lys-63–linked ubiquitin chains contribute to signaling (150–152).

Hsp70s are modified by several undisclosed E3 Ub ligases at multiple residues (Figs. 1–3). Most of these sites were identified in high-throughput Ub-proteomics studies, and the functional implications of these modifications remain largely unclear (153–156). The best-understood Ub ligase-Hsp70 interactions are CHIP-Hsp70/Hsc70 and Parkin-Hsp70/Hsc70. CHIP binds to Hsp70 and Hsc70 and modifies their client proteins as well as Hsp70/Hsc70 itself. Whereas most studies suggest that polyubiquitination of Hsp70 and Hsc70 promotes their proteasomal degradation, other work did not find supporting evidence for this process (157–161). CHIP-conferred Ub arrays on Hsp70 and Hsc70, while substantially overlapping, differ in the modification of specific sites (*e.g.* Lys-159 in Hsc70 and its orthologous residue in Hsp70) and the length and branching of the added Ub chains (161), indicating that a single E3 ligase can imprint different Ub patterns on distinct substrates. Parkin is a Parkinson's disease–associated E3 Ub ligase that monoubiquitinylates Hsp70 and Hsc70 on multiple sites (162). Interestingly, monoubiquitination of Hsp70 and Hsc70 by Parkin does not alter their turnover, steady-state levels, or proteasomal degradation, suggesting a role for ubiquitination in signaling transduction or chaperone regulation beyond degradation.

The ubiquitin-mediated degradation of several important proteins is regulated by other nearby PTMs. For example, phosphorylation of Thr-286 on cyclin D1 promotes its degradation and correct cell cycle progression (163). It is interesting to note that several PTMs exist in close proximity to identified sites of Hsp70 ubiquitination (see Figs. 1–3). Given the number of PTMs on Hsp70, it is also possible that ubiquitination acts as a reset button for the chaperone code. While currently just speculation by the authors, it is feasible that once a certain number/combination of modifications has been reached, ubiquitination may promote chaperone destruction, with newly synthesized unmodified Hsp70 taking its place.

Hsp70 thiol oxidation

Thiol oxidation occurs on cysteines that are modified under conditions of oxidative stress or under exposure to thiol chelators or oxidizers, perhaps as an oxidative stress signal (164–166). This can lead to new disulfide linkages on the same protein or between proteins, formation of new moieties (including sulfenic acid and glutathionylation), and electrophilic adduction (164, 167, 168). In yeast cells, Ssa1 and Ssa2 are subject to thiol oxidation on Cys-15, Cys-264, and Cys-303 when treated with thiol-reactive compounds (169). Mutation of these sites prevents Hsf1 activation in response to oxidative agents. Several of these sites are conserved in mammalian cells and are also oxidized. Human Hsp70 (but not Hsc70) is also oxidized on Cys-267 and Cys-306, which inhibits its ATPase activity (71). This appears to be a primary mechanism of action for the anti-Tau drug methylene blue and suggests that going forward, compounds that manipulate Hsp70 oxidation status may be clinically relevant.

The above studies suggest a fascinating mechanism by which Hsp70 is a direct sensor in the cellular response to oxidative stress (164). It appears that other Hsp70 isoforms can also perform this function. The yeast BiP ortholog Kar2 is oxidized on Cys-63 under ER oxidative stress, which is reversed via one of Kar2's nucleotide exchange factors, Sil1, apparently via thiol-disulfide exchange between the oxidized Cys-63 of Kar2 and the Cys-52 and/or Cys-57 of Sil1 (165, 167, 170). Sulfenylation and glutathionylation diminish Kar2's ATPase activities while leaving its peptide binding activities intact, converting Kar2 from an ATP-dependent foldase into an ATP-independent holdase (167, 170). This modification is cytoprotective during oxidative stress, but not during nonstress conditions (167, 170). Recent work suggests that Cys-574 and Cys-603 of HSP70 can also undergo a novel type of modification (glutathionylation) that promotes *in vitro* an increase in ATPase activity but decreased interaction with HSF1 in HeLa cells (166). Although

further studies are needed, this suggests that glutathionylation in response to oxidative stress may also contribute to the cellular response to oxidative stress though HSF1 activation. In human U2OS bone cells, the peroxidase GPx7/NPGPx interacts with BiP under oxidative stress, and *in vitro* mediates the formation of a disulfide bond between BiP Cys-41 and Cys-420 (169). The formation of this bond promotes increased BiP activity, ER protein folding, and ER oxidative stress resistance (134). Although this disulfide bond formation was not shown *in vivo*, other proteins, including the peroxide-detoxifying enzyme PRDX-2, have been shown to form disulfide bonds in *C. elegans* in response to H_2O_2 (171–173), demonstrating oxidized disulfide bond formation *in vivo* and suggesting the possibility that Hsp70 family members that form disulfide bonds in yeast cells during stress response may also do so in other eukaryotic organisms (169). In *C. elegans*, Cys-307 of the cytosolic Hsc70 ortholog HSP1 is oxidized when H_2O_2 detoxification is impaired, during induced H_2O_2 stress, and during development (172, 173).

Other PTMs on Hsp70 family proteins

High-throughput proteomics studies continuously expand our understanding of which PTMs contribute to the chaperone code (155, 174). Succinylation is an emerging PTM in which a succinyl group is reversibly linked to available lysine residues. The mapping of succinylation sites in *S. cerevisiae*, human (HeLa) cells and mouse liver tissue demonstrated the presence of this modification on HSPA1A, HSPA5, HSPA8, and Ssa1, yet the functional implications of these modifications remain unknown (155, 175). SUMOylation refers to the covalent linkage of a small ubiquitin-like protein (SUMO) to exposed lysine residues. HSPA1A, HSPA5, and HSPA8 are all SUMOylated, but, similar to succinylation, the functional consequences of Hsp70 succinylation are elusive (174, 176, 177). NEDDylation, a modification in which the ubiquitin-like protein, NEDD8, is reversibly attached either singly or in a NEDD8-chain to a Lys residue on a target protein has also been shown to occur on Hsp70s (178, 179). NEDD8 attaches to the ATPase domain of Hsp70 between amino acids 190 and 394 (178). In human U2-OS cells, de-NEDDylation is required for Hsp70 to be released from APAF1 and allow APAF1 to participate in the initiation of apoptosis (178). Interestingly, the presence of mono-NEDD8 stimulates, whereas NEDD8 chains inhibit, Hsp70 ATPase activity (178).

Conservation of PTM sites between Hsp70 isoforms

An unanswered question in chaperone research is why cells express so many highly related and apparently functionally redundant Hsp70s. The historical model is that cells express the Hsc70 isoform constitutively to maintain homeostasis and express inducible Hsp70 variants under stressful conditions to assist in the additional proteotoxic burden. Recent studies clearly demonstrate that human Hsp70 variants display differential preferences for clients and co-chaperones (180, 181). These studies are corroborated by functional studies in yeast that reveal phenotypic differences in yeast expressing single Ssa isoforms (23, 182–185). Taken together, the data suggest that cytoplasmic Hsp70 variants have overlapping but distinct client-binding specificities driving unique roles in the cell.

PTMs on Hsp70 isoforms fall into distinct categories: those that are highly conserved and maintained throughout different organisms and those where the site is not conserved. It is probable that sites that are conserved throughout evolution are involved in important and fundamental cellular processes. Examples can be found in three phosphorylation sites discussed in this review, Thr-38, Thr-504, and Thr-636 (Hsc70 numbering). Thr-38 regulates the cell cycle, Thr-504 regulates the Hsp70 monomer-dimer balance, and Thr-636 determines whether a client is refolded or targeted for degradation (57, 58, 186). The conservation of Thr-504 (and surrounding amino acids) is particularly fascinating as it suggests that dimerization of all Hsp70 isoforms (as either homodimers or heterodimers of two different isoforms) is possible.

As discussed throughout this review, several sites of PTM are not conserved between isoforms (Fig. 3). For example, Lys-562 is only present in constitutively expressed Ssa1 and Ssa2 and not in Ssa3 and Ssa4. In times of heat shock, Ssa3 and Ssa4 are induced to respond to the additional proteotoxic burden on the cell. However, this response relies on the relatively slow pace of transcription and would not be fast enough for a cell to deal with acute heat stress. It is our belief that in the initial stages of heat shock, the deacetylation seen on Ssa1 and Ssa2 temporarily switches their function to be closer to that of Ssa3 and Ssa4 to maintain cell viability until enough Ssa3 and Ssa4 has been translated. Whereas the idea that Hsp70 PTMs can switch the functions of isoforms from one to another may seem farfetched, it is important to note that chaperone isoforms differ by a few amino acids. Previous work on yeast established that a single amino acid change can switch prion-related functions of Ssa1 to that of Ssa2 (184). The position of this amino acid, Ala-83, is interesting because the equivalent residues in Hsp70 and Hsc70 are both serines previously detected as phosphorylated residues (Fig. 3). Ssa3 possesses a threonine at this site and may also be modified. Whereas phosphorylation of Hsp70 and Hsc70 at this site may alter functionality in mammalian cells, perhaps the Ssas have diverged in sequence to produce isoforms with distinct cellular functions. Other less well-examined examples of site diversification exist. Early studies identified that Hsp70 was phosphorylated upon heat shock at Tyr-525 (187). Tyr-525 is a "hinge" residue that may alter C-terminal lid closure. Similar to the previous example, this tyrosine is present in Hsp70, Hsc70, and Ssa3 but is nonphosphorylatable phenylalanine in Ssa1, Ssa2, and Ssa4 (Fig. 3). Although further studies are needed, the authors speculate that variation in sites like this may also be a way of controlling stoichiometry of a modification. If a PTM site is totally conserved, then all Hsp70s present will be modified on this site at one time. In contrast, if only certain isoforms have a particular PTM site, then smaller pools of chaperones will be altered by this modification.

Primary mechanisms of action of the chaperone code

There are clearly a large number of different PTMs present on Hsp70 proteins. A major question remains: What are the majority of these modifications doing? Hsp70 function is

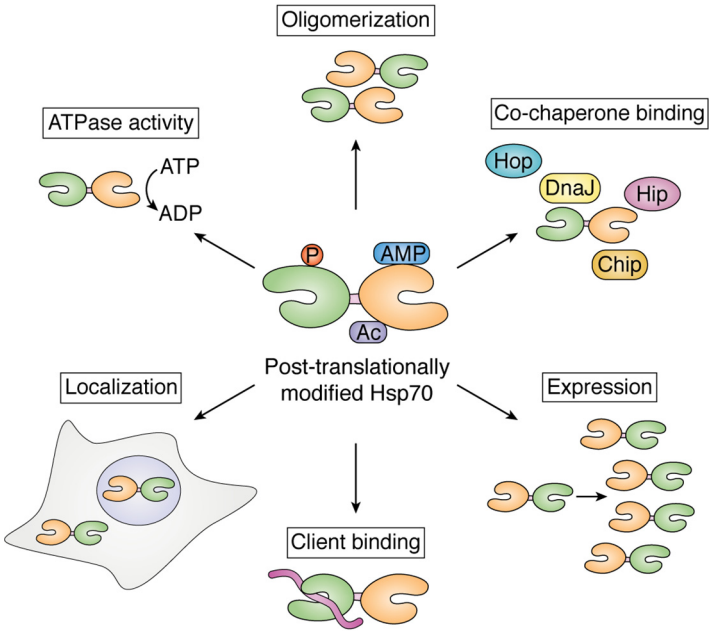

Figure 4. The hallmarks of Hsp70 regulation. This illustration encompasses six regulatory processes (*outside wheel*) that are affected by the post-translational modification of Hsp70: co-chaperone binding, expression, client binding, localization, ATPase activity, and oligomerization. Most of these processes are interdependent (*e.g.* ATPase activity and client binding).

regulated through several different processes including co-chaperone binding, transcription, expression of related isoforms, cellular localization, client specificity and self-interaction ((2) and Fig. 4). It is clear that many of these regulatory mechanisms are tied to one another. J-proteins bind and stimulate the ATPase activity of Hsp70; different Hsp70 isoforms have different expression patterns, ATPase activities and client specificities. It remains challenging to tease out the primary roles of Hsp70 PTMs from existing studies. For example, the monomer-dimer ratio of both Hsp70 and the ER-resident BiP proteins appears to be regulated by phosphorylation and acetylation (82, 186), but the roles for co-chaperones and effect on client specificity and whether this process allows heterodimerization of Hsp70 isoforms remains unclear (36). Likewise, the dynamic shuttling of Hsp70 to the nucleus post-heat stress is at least in part regulated by phosphorylation and AMPylation events: phosphorylation or phosphomimetic mutations of Tyr-525 increase their nuclear accumulation, whereas phosphoinhibitory mutations retain Hsp70-1 in the cytoplasm (187). Similarly, increased Hsp70 AMPylation prevents its nuclear shuttling following heat stress (101). The NBD is the site of ATP hydrolysis and the interacting region for several co-chaperone proteins. We believe that the majority of Hsp70 PTMs on the N terminus are directly impacting Hsp70 structure to alter ATPase activity (either directly through ATPase site rearrangement or indirectly through co-chaperone binding). On the other hand, PTMs on the C terminus are much more likely to direct Hsp70-client specificity, folding, and release.

Conclusions and future perspectives

Hsp70s have been heavily researched for decades, resulting in several thousand publications on their mechanisms of action and their roles in the cell. Improvements in -omics technologies have resulted in the detection of a large number of Hsp70 PTMs, the majority of which have no known function. Linking these PTMs to chaperone function and determining the stresses/enzymes that regulate these specific sites remains a major challenge for understanding the chaperone code. Many of the existing studies have taken a "bottom-up" approach, mutating individual modified residues on Hsp70s to prevent PTM addition and analyzing the effect on both *in vitro* activity (refolding capability, structural changes, and ATP hydrolysis) and physiological relevance (client specificity, cellular localization, co-chaperone interaction). While these approaches have been effective, it is important to remember that it is common for multiple PTMs on proteins to be simultaneously activated in response to cellular stresses. It is thus also useful to take a "top-down approach," where global PTMs on Hsp70 are examined under stress conditions.

The number of currently identified PTMs on Hsp70 family proteins appears at first glance to be shockingly high. For Hsc70, 60% of the total Ser, Thr, and Tyr residues have been identified as phosphorylated (from the GPM database). However, to put this into perspective, this value is comparable with that for another major chaperone, Hsp90 (56%) and the metabolic enzyme GAPDH (62%). There are other proteins in the cell that have a much higher proportion of modified residues (*e.g.* the DNA damage response protein 53BP1 (93%)). At this time, it is hard to know how many of these sites are functionally relevant. Some of these sites may be false positives arising from the misinterpretation of low quality MS spectra by commonly used analysis software. Nevertheless, many of the modified amino acids are well-conserved throughout evolution, suggesting regulatory importance. Some sites may become important only in the context of other site modifications, making standard single-site mutagenesis ineffective.

Where will the future of chaperone code research take us? In the coming years, we might expect further studies to focus on determining how individual sites on Hsp70s are regulated and their impact on Hsp70 function, particularly in the context of cancer and neurodegeneration. Although phosphorylation, methylation, acetylation, AMPylation, NEDDylation, and ubiquitination have been detected on Hsp70, over 100 different PTMs have been identified on other proteins. As the resolution of proteomic technologies improves, we may thus find that Hsp70s are modified with many more PTMs than currently known. Studies of individual sites are still nontrivial, especially given the complex reciprocal relationship between chaperones and those clients that are able to modify chaperones and that choice of expression host dictates PTMs added to Hsp70 when made recombinantly (57, 188, 189).

A greater challenge will be understanding how multiple PTMs on Hsp70 interact and cross-talk. Several identified PTMs on Hsp70 family proteins modify the *same* amino acid and are thus mutually exclusive. For example, BiP Thr-518 can be modified by either phosphorylation or AMPylation. It is likely in this case that AMPylation prevents Thr-518 phosphorylation, holding BiP in an inactive state until needed by the cell. Lysine residues can also be modified by a wide range of PTMs, including ubiquitination, acetylation, and methylation. Similarly, several lysines on Hsp70 family proteins have been identified as modified in multiple ways. For example, Lys-246 and Lys-601 on Hsp70 and Lys-268 and Lys-585 on BiP are modified by acetylation, methylation, and ubiquitination (Fig. 1). These mutually exclusive modifications may be activated by opposing signaling pathways in response to distinct cellular cues. The presence of multiple PTMs on single sites makes analysis more complex; for example, site-directed mutation of an amino acid would prevent *all modifications* occurring. It is interesting to note that there are several areas on Hsp70 where clustering of PTMs occur (*e.g.* amino acids 245–255, 275–285, 420–440, and 490–500) (see Figs. 2 and 3). It is highly possible that some PTMs act in synergy and are required for others to be added, as in the case seen for the FNIP1 co-chaperone (190). In contrast, some PTMs may be antagonistic, particularly if they exist in close proximity. Many writer enzymes, such as kinases and acetyltransferases, have very specific substrate sequence requirements, and modification of the surrounding residues may prevent other PTMs from occurring.

If we think of the total sum of the modifications on a single Hsp70 "proteoform" as a complex code in response to internal and external cues, then the overall population of these proteoforms will reflect the overall health and status of a cell. The temporal and spatial resolution of Hsp70 proteoforms will be a major undertaking. Current proteomic technologies require the digestion of proteins to peptides, which destroys information about the combinations of PTMs present on a Hsp70 proteoform. Newer methodologies, however, such as top-down proteomics, should mitigate these issues, although the large size of Hsp70 makes this kind of analysis currently challenging (191).

Even when this level of complexity is resolved, we will have to contend with the multiple isoforms of Hsp70 in the cell. It is highly likely that different Hsp70 isoforms possess unique PTMs suited for their individual function. More intriguing is the possibility that PTMs are present on Hsp70s that allow instantaneous conversion of one isoform's function to another under particular stresses. This situation is further complicated by evidence that Hsp70 can form dimers, trimers, and higher-order oligomers, the formation of which is also linked to Hsp70 PTMs (36).

Finally, even when all of the mechanisms and physiological relevance of Hsp70 PTMs have been understood, these models will have to be incorporated into the understanding of the entirety of the chaperone code. Many other important chaperones, including Hsp90, Hsp104, and Hsp60, and co-chaperones, such as Hsp40 and CHIP, are highly modified, many of which influence their interaction with Hsp70. The understanding of this bewildering array of chaperone PTMs will take a concerted effort among researchers and should cement chaperones as the cross-roads for major signaling events in the cell.

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Abbreviations—The abbreviations used are: Hsp, heat shock protein; PTM, post-translational protein modification; NBD, nucleotide-binding domain; SBD, substrate-binding domain; ER, endoplasmic reticulum; UPR, unfolded protein response; ART, ADP-ribosyltransferase; PARP, poly(ADP-ribose) polymerase; NEF, nucleotide exchange factor; ERK, extracellular signal–regulated kinase; RFC, reduced folate carrier protein; SOD2, superoxide dismutase; Ub, ubiquitin; SUMO, small ubiquitin-like protein.

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